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Restoration of Antibiotic Sensitivity via Plasmid Curing: The Effect of *Nigella sativa* on Multidrug Resistant *Staphylococcus aureus*

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Abstract

The global rise in multidrug resistant (MDR) bacterial infections poses significant challenges to the public health especially the hospital environments which demand innovative solutions. This study investigates and evaluates the plasmid curing potential of *Nigella sativa* seed extract on multidrug resistant *Staphylococcus aureus* isolated from clinical samples. The seed extract of *N. sativa* was screened for plasmid curing potential by exposing the isolates to standard antibiotics and subjecting them back to the standard antibiotics after exposure to the extract of *N. sativa* for reassessment. Initial antibiotic sensitivity testing confirmed high resistance, especially to amoxicillin (91.67%) and rifampin (91.67%). The isolates were treated with subminimum inhibitory concentrations (50-200µl/ml) of *N. sativa*. The post-curing antibiogram of *S. aureus* treated with *Nigella sativa* revealed significant plasmid curing activity, with improved sensitivity to Levofloxacin 11(91.67% cured), Ciprofloxacin was up to 58.33%

sensitive and Amoxicillin was 4(33.33%). Two ways ANOVA confirmed the effect of *N sativa* concentration on sensitivity restoration was statistically significant (p=0.016). The findings of this study provide insight into the role of plasmid in MDR pathogens and the potential of plant-based agents in combating plasmid mediated resistance.

Keywords: *Plasmid curing, N sativa, S aureus, and Antimicrobial resistance*

Introduction

Resistance to antibiotics may come from parent bacteria however they can as well pick up resistance traits from their competitors (Ringel *et al.,* 2016). Some bacteria have the capacity to inject toxic cocktail into their competitors causing cell lysis and death by integration of genetic materials. These organisms may be carrying drug resistance genes that may be transferred into predator by uptake of DNA fragment taken up which has been released by the prey which signifies that genes for such resistance can be transferred from one bacterium to another (Barrow & Feltham, 2003; Ringel *et al.,* 2016; Zaman & Chawdhury, 2024).

Plant are known to have a direct effect on microbial life (Bislava *et al.,* 2021) therefore, using plants for plasmid curing is a future driven success. The plant has been predicted to be a promising solution to plasmid-mediated antibiotic resistance (Adeboye & Manga, 2022). Plasmid targeting for curing is a promising novel drug if successful, because the most essential principal feature of a drug target is for the site not to be analogous to eukaryotic cell, of which only yeast cells are eukaryote that acquires plasmid and no multicellular organism has that feature of plasmid being in its cell. This paper focuses on curing plasmid in multidrug resistant clinical isolates of *Staphylococcus aureus* using organic extracts of *Nigella sativa* (black seed).

Statement of the Problem

Considering the natural and accidental discovery of antibiotics, bacterial infections should be treatable with antibiotics but resistance has become a common phenomenon preventing the cure of infections which have various mechanisms of actions against antibiotics and therefore remains a problem. The antibiotics of different structures, forms, modes of action have been developed to cure bacterial diseases but all are almost exhausted due to bacterial resistance which are mostly as a result of

alterations in either cellular, physiological or genetic makeup, leaving the infection to persist (Sekhi *et al.,* 2021). Such line of drugs have targeted many cellular organelles and other components of the cell but a non-chromosomal DNA known as plasmid contributes to such resistance and render a sensitive bacteria cell drug-resistant by empowering such bacteria host with the ability to resist drugs using genes coded on the plasmid that confer resistance. (Zaman & Chawdhury, 2024).

Justification

Since plasmid has been identified as a problem in the course of antimicrobial resistance (AMR), there is a need to cure plasmids and reassess sensitivity following the curing of the plasmid as an identified problem in order to improve the sensitivity of bacteria to antibiotics. The known curing agents like ethidium bromide cannot be transcribed to human use because of their ability to cause gene mutations as they are known intercalating agents that are dangerous for human use. Therefore, there is need to explore organic compounds with minimum health risk as replacement for chemicals like the aforementioned, such as acridine orange and bromide. It will also be a great model to block the activity of such plasmid genes capable of rendering antibiotics inactive or ineffective (Rithanya & Ezhilarasan, 2021).

The aim of the study is to carry out Plasmid Curing on Multi-drug Resistant *Staphylococcus aureus* with the specific objectives of plasmid curing of MDR *S aureus* using *Nigella sativa* extracts as curing agents and reassess for sensitivity to the antibiotics the isolates were previously displaying resistance to.

Materials and Methods

Collection of Plants for Plasmid Curing

The seeds of *Nigella sativa* (Black seed, Abbatu sauda) was purchased from herbal store in Sokoto Metropolis and authentication was done in Usmanu Danfodiyo University, Sokoto Biological Science Herbarium with accession number UDU/ANS/0106.

Screening of Extracts of *N sativa* for Antibacterial Activity

Prior to MIC, the screening of extracts of *N sativa* for antimicrobial activity were confirmed using an agar well diffusion with crude extract according to the method of Oyeleke & Manga (2008). For the zone of inhibition, *Nigella sativa* crude of 0.2ml was dispensed in dished borrow of solidified Mueller Hinton Agar.

Soxhlet Extraction of *Nigella sativa*

Soxhlet extraction is a classical solid-liquid extraction procedure and was carried out using the organic solvent n-hexane. It has the advantage of large-scale extraction and high extraction yields. This process was performed according to Hu et al. (2021). All the seed samples were dried to remove moisture to allow penetration of the organic solvent, which has lower density compared to water. A mortar and pestle were used to reduce the seed size to increase surface area. The black seeds were extracted using the Soxhlet extraction process described by Lekgari (2015) and Garba et al. (2021) as follows: A quantity of 250 ml of the organic solvent n-hexane was measured and poured into a round-bottom flask equipped with a Soxhlet apparatus and condenser. Twenty grams (20 g) of the *Nigella sativa* sample were placed inside the thimble extractor and repeated four times. The Soxhlet extraction was then carried out at 55°C for 1 hour. At the end of extraction, the oil-solvent mixture was initially separated using a rotary evaporator and then further dried in an oven. Extraction was continued until the oil was completely removed from the seeds.

Gas Chromatography–Mass Spectrometry (GC-MS) of Extracts

GC-MS analysis was carried out according to the description by Omoregie et al. (2015), with antimicrobial compound extraction following the method by Canli et al. (2016). The GC-MS analysis of the *N. sativa* extract was performed using a CasLac GC-MS (Agilent Technologies Autosystem GCMS-QP2010 PLUS, Shimadzu), equipped with a VF-5ms fused silica capillary column (30 m length, 0.25 mm diameter, and 0.25 μm film thickness). For GC-MS detection, an electron ionization system with an ionization energy of 70 eV was used. Helium gas (99.99%) was used as the carrier gas at a constant flow rate of

1.51 ml/min. The injector and mass transfer line temperatures were set at 200°C and 240°C, respectively. The oven temperature was programmed from 70°C to 220°C at 10°C/min, held isothermal for 1 minute, and then raised to 300°C at 10°C/min. Two milliliters of aqueous sample solution were manually injected in splitless mode, with a split ratio of 1:40 and a mass scan range of 50–600 amu. The total GC-MS run time was 35 minutes. The relative percentage of extract constituents was expressed based on peak area normalization. The spectra of the compounds were compared with those in the National Institute of Standards and Technology (NIST) library database. Tests for phytochemical compounds in the plant extracts viz. steroids, triterpenoids, alkaloids, tannins, flavonoids, diterpenes, glycosides, and saponins were conducted according to the standard method (Kumar et al., 2009).

Determination of Minimum Inhibitory Concentration (MIC) of the Plant Extract

The Minimum Inhibitory Concentration (MIC) of the extract against *S. aureus* was determined using the broth dilution method (Cheesbrough, 2006; Wiegand et al., 2008). The MIC is defined as the lowest concentration of extract that inhibited visible growth. The tube dilution method was employed. The *N. sativa* oil extract was serially diluted to obtain varying concentrations of 400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5 mg/ml in Mueller-Hinton broth containing Tween 80.

A standardized suspension of *S. aureus* was prepared according to the McFarland standard, typically 10^8 CFU/ml. Aliquots of 2 ml of the prepared Mueller-Hinton broth were introduced into sterilized test tubes, cooled, and inoculated with 5 µl of the McFarland-standardized bacterial suspension. A series of tubes containing different concentrations of the *N. sativa* extract in a doubling dilution series was made. The *S. aureus* isolates (039SA/181SA) were inoculated into each prepared test tube. The tubes were incubated at 37°C for 24 hours. Growth was observed by assessing turbidity. The MIC was the lowest concentration of the antimicrobial agent that inhibited growth (Levine, 2018).

Plasmid Curing

The plasmid curing procedure was performed as described by Gunjal et al. (2020), with modifications to suit the design of this study. Cultures of *S. aureus* were grown in Mueller-Hinton broth in the presence of *Nigella sativa* plant extracts at sub-inhibitory concentrations of 200, 100, and 50 µl/ml for 24 hours at 37°C. The cultures were subcultured onto nutrient agar to obtain well-isolated colonies of cured isolates. Acridine orange (10 µg/ml) in Mueller-Hinton broth was used as a positive control, while an untreated bacterial culture in MHB served as a negative control. The bacteria grown in the extract were subcultured after 24 hours for reevaluation of antimicrobial sensitivity. Strains that failed to grow in the presence of antibiotics were considered putative cured derivatives. The curing response rate was subjected to sensitivity reevaluation to assess the curing efficiency of the plant extracts.

Antimicrobial Activity Test after Curing

Curing required growing the resistant bacteria in the subminimum inhibitory concentration of each extract and then subculturing on freshly prepared nutrient agar before assessing antibacterial activity against the previously resisted antibiotics. Aliquots of 0.1 ml of inoculum suspension, standardized using the McFarland method, were lawn cultured on Mueller-Hinton agar plates using the disc diffusion method as previously applied during the initial antibiogram testing. The plates were left for 30 minutes to allow diffusion. Antibiotic discs were then placed onto the MHA plates. The plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured to determine post-curing sensitivity, recorded in millimeters as the zone of inhibition (Muscara et al., 2021).

Bio-centrifugation for Plasmid DNA Extraction

Plasmid extraction was performed according to Altamirano et al. (2021), with slight modifications based on the manufacturer's instructions (HiYield). Bacterial cells were grown for 24 hours, washed in nuclease-free water, and centrifuged. The supernatant was discarded, leaving the cell pellets. HiYield reagents for plasmid DNA extraction were used, following the method described by Laguerre et al. (1992). The plasmid

curing capacity of *N. sativa* was assessed using agarose gel electrophoresis to visualize DNA band patterns.

Agarose Gel Electrophoresis of the Plasmid DNA

One gram (1.0 g) of agarose powder was dissolved in 100 ml of 1× Tris-Borate-EDTA (TBE) buffer in a 500 ml Erlenmeyer conical flask. The flask was swirled to dissolve the agarose powder and heated in a microwave oven until a clear molten solution was obtained. The molten agarose was allowed to cool on the bench, and 5 µl of ethidium bromide (an intercalating agent) was added to the gel for visualization of DNA fragments. A comb was placed in the casting tray to form wells for loading the amplicons. The gel was submerged in 1× Tris-EDTA running buffer in the electrophoresis chamber. PCR products were mixed with loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol). The first and last wells were loaded with 1 kb molecular weight DNA ladders to estimate the sizes of resulting DNA fragments. The electrophoresis chamber was connected to a power source and run at 100 V (6 V/cm) for 40 minutes. The DNA bands were visualized using UV light illumination (Lee et al., 2012).

Results

Table 1 shows the zone of inhibition exhibited by *S. aureus* against standard antibiotics before plasmid curing, indicating over 90% resistance to amoxicillin and rifampin.

Table 2 presents the zones of inhibition for antimicrobial screening of crude extracts of *N. sativa*. The crude extract was screened on isolates 039SA and 181SA for antimicrobial activity. *N. sativa* yielded a zone of inhibition of 15 mm on 039SA and 14 mm on 181SA.

Table 3 shows the minimum inhibitory concentration (MIC) of *N. sativa* extract at 25 mg/ml on *S. aureus* isolates 039SA and 181SA.

Table 4 presents the *Nigella sativa* GC-MS analysis, highlighting 6 of the 72 detected compounds, along with their peaks, retention times, chemical names, and 3D structures.

Table 5 shows the post-curing antibiogram pattern following treatment with *N. sativa* at concentrations of 50 µl, 100 µl, and 200 µl. The results demonstrate variability in antibiotic efficacy and the effect of *N. sativa* concentration on *S. aureus* susceptibility. A two-way ANOVA was

conducted. The p-value for the effect of antibiotic type was 1.000, indicating no significant effect, while the concentration of *N. sativa* showed a significant effect ($p = 0.016$). This implies that variations in *N. sativa* concentration had a statistically significant impact on the response of cured *S. aureus* isolates to previously resisted drugs.

Table 1: Plasmid Positive *Staphylococcus aureus* Pre Curing Antibiogram Pattern against Standard Antibiotics

Antibiotics	**Sensitive**	**Intermediate**	**Resistant**
Erythromycin	0(0)	4(33.33)	8(66.67)
Levofloxacin	2(16.67)	1(8.33)	9(75)
Ciprofloxacin	2(16.67)	2(16.67)	8(66.66)
Gentamycin	6(50)	1(8.33)	5(41.67)
Amoxicillin	0(0)	1(8.33)	11(91.67)
Streptomycin	0(0)	7(58.33)	5(41.67)
Rifampin	0	1(8.33)	11(91.67)

Table 2: Extracts Antimicrobial Screening based on Zone of Inhibition

Tested Isolates	*N sativa*
039SA	15mm
181SA	14mm

Table 3: Minimum Inhibitory Concentration (MIC) of *Nigella sativa* extract Against *S aureus* (SA)

Bacterial Isolate	**Organism Type**	**MIC of *N sativa***
039SA	*Staphylococcus aureus*	25mg/ml
181SA	*Staphylococcus aureus*	25mg/ml

Table 4: Gas Chromatography Mass Spectrometry Analysis of *Nigella sativa*

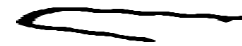
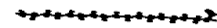
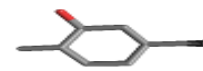
S/ No	Peak	Retention Time	Compound Nomenclature	Common Name	3D Chemical Structure
1	44	20.466	Oleic acid	Oleic acid	
2	45	20.503	Octadecanoic acid	Stearic acid	
3	29	19.278	N-Hexadecanoic acid	Palmitic acid	
4	51	21.232	11,14-Eicosadienoic acid	Arachidonic acid	
5	30	19.308	Hexadenoic acid, ethyl ester	Ethyl caproate	
6	67	23.055	1-Cyclohexyldimethylsilyloxy-2-methyl propane	dihydrocarveol	

Table 5: Antibiogram profile of *Staphylococcus aureus* Post-Plasmid Curing with *Nigella sativa* Against Standard Antibiotics

Antibiotics	Antibiogram of the isolates (%)			
	***N sativa* Concentration (ug)**	**Sensitive**	**Intermediate**	**Resistant**
Erythromycin	50	1(8.33)	3(25)	8(66.67)
	100	2(16.67)	0(0)	10(83.33)
	200	3(25)	3(25)	6(50)
Levofloxacin	50	7(58.33)	0(0)	5(41.67)
	100	7(58.33)	0(0)	5(41.67)
	200	7(58.33)	0(0)	5(41.67)
Ciprofloxacin	50	6(50)	1(8.33)	5(41.67)
	100	6(50)	0(0)	6(50)
	200	7(58.33)	0(0)	5(41.67)
Gentamycin	50	8(66.67)	0(0)	4(33.33)
	100	8(66.67)	0(0)	4(33.33)
	200	7(58.33)	0(0)	5(41.67)
Amoxicillin	50	0(0)	1(8.33)	11(91.67)
	100	1(8.33)	0(0)	11(91.67)
	200	2(16.67)	1(8.33)	9(75)
Streptomycin	50	3(25)	6(50)	3(25)
	100	1(8.33)	7(58.33)	4(33.33)
	200	3(25)	3(25)	6(50)
Rifampin	50	0(0)	2(16.67)	10(83.33)
	100	0(0)	1(8.33)	11(91.67)
	200	0(0)	1(8.33)	11(91.67)

Discussion

The resistance pattern of the plasmid-positive *S. aureus* revealed that 91% of the isolates were resistant to amoxicillin and rifampin, followed by levofloxacin (75%) and erythromycin (66.67%). This is similar to the resistance range of 75–91% reported by Jam-Kmeicik et al. (2025). None of the *S. aureus* isolates was sensitive to streptomycin, erythromycin, amoxicillin, or rifampin, though various intermediate resistances were observed. With the increasing prevalence of multidrug-resistant bacteria globally, *Staphylococcus aureus* poses a significant public health threat (Okoye et al., 2022). Its resistance is often plasmid-mediated (Almuhayawi et al., 2023).

The GC-MS (Gas Chromatography–Mass Spectrometry) profiling of *Nigella sativa* seed extract revealed six key bioactive compounds out of 72 detected, which may significantly contribute to its plasmid-curing and antimicrobial effects. These include oleic acid, stearic acid (octadecanoic acid), palmitic acid (n-hexadecanoic acid), arachidonic acid (11,14-eicosadienoic acid), ethyl caproate (hexadecanoic acid, ethyl ester), and a dihydrocarveol derivative.

Oleic acid, a monounsaturated omega-9 fatty acid, is known for its ability to disrupt bacterial membranes and enhance the permeability of other antimicrobial agents. It has also been linked to efflux pump inhibition, which is a key mechanism in antibiotic resistance (Obukhova & Murzina, 2024). Oleic acid nanoparticles have shown potential for use in antimicrobial treatment (Ibrahim et al., 2024).

Stearic acid, a saturated fatty acid, possesses antibacterial and anti-inflammatory properties and may destabilize bacterial membranes or interfere with plasmid maintenance. Palmitic acid is another antimicrobial fatty acid reported to bind plasmid DNA and potentially block plasmid replication by affecting DNA supercoiling and relaxation (Olivia et al., 2021). Dihydrocarveol derivatives are known for strong antimicrobial and antifungal activity, possibly disrupting plasmid retention or replication through oxidative stress.

These compounds, individually or in combination, appear to disrupt plasmid stability and replication, which supports the observed restoration of antibiotic sensitivity following curing. They may inhibit plasmid replication by intercalating into DNA or disrupting replication machinery (Obukhova & Murzina, 2024; Zhu et al., 2024). The combined action of multiple fatty acids creates an intracellular environment hostile to plasmid-bearing *S. aureus*, promoting the loss of plasmid-mediated resistance.

The inhibition zones (Table 2) demonstrate the broad-spectrum antimicrobial potential of the crude extract, while the GC-MS data provide a chemical explanation for this activity. The large inhibition zones (≥14 mm) are notable for a crude extract and indicate strong bioactivity, highlighting the therapeutic potential of *N. sativa*.

The agar well diffusion assay showed measurable inhibitory zones against multidrug-resistant *S. aureus* strains 039SA and 181SA (15 mm and 14 mm, respectively). These results justify the investigation of *N. sativa* as a plasmid-curing agent and are consistent with the findings of Garba et al. (2021), who reported similar antibacterial activity of *N. sativa* against multi-antibiotic-resistant diarrheagenic bacteria.

Altogether, the results support the hypothesis that the antimicrobial and plasmid-curing effects of *N. sativa* stem from its phytochemical composition, rich in functional lipophilic compounds that disrupt bacterial membranes, induce stress responses, and interfere with plasmid-encoded resistance mechanisms.

In summary, *N. sativa* showed notable antimicrobial activity against MDR *S. aureus* isolates, supported by its GC-MS profile, which identified multiple antimicrobial fatty acids and terpenoids. These findings establish *N. sativa* as a credible candidate for natural plasmid-curing therapies aimed at reversing antibiotic resistance. Its biochemical profile supports its observed effects in curing plasmids and restoring drug sensitivity.

Thymoquinone, a major compound in *N. sativa*, is well-documented for its antibacterial properties (EiAlfy, 1974; Paarakh, 2010; Samkelo, 2017). In this study, thymoquinone's role is evident in the curing of plasmid-mediated resistance genes. Its presence supports earlier findings by Samkelo (2017). However, the MIC of 25 mg/ml found in this study contrasts with Garba et al. (2021), who reported an MIC of 100 mg/ml.

Effective plasmid curing helps restore bacterial susceptibility and re-establishes the clinical usefulness of antibiotics that might otherwise be abandoned. Ciprofloxacin resistance patterns were similar to levofloxacin, and curing success was also comparable—91.67% (11 out of 12 isolates). Interestingly, the 50 µl concentration outperformed the 200 µl extract, curing 6 strains versus 5.

The curing of plasmids is a promising strategy for restoring antibiotic susceptibility in MDR organisms. In this study, *Nigella sativa* extract showed effective plasmid-curing potential against MDR *S. aureus*. Pre-curing sensitivity tests revealed widespread resistance, especially to amoxicillin and rifampin (over 90%).

After curing, levofloxacin and ciprofloxacin resistance were reversed in more than 50% of isolates across all concentrations. Gentamycin resistance was reversed in four isolates at 100 µl and 200 µl concentrations. Amoxicillin-resistant strains, although common, showed less pronounced change post-curing, with only 4 isolates (33.33%) reverting to sensitivity.

Lower concentrations, particularly 50 µl/ml, were surprisingly more effective in some cases. Ciprofloxacin sensitivity increased to 58.33%, and levofloxacin showed consistent sensitivity (58.33%) across all concentrations. Rifampin, on the other hand, showed little change, suggesting a need for higher concentrations or combination therapies.

Statistical analysis showed that concentration had a significant effect ($p = 0.016$), while antibiotic type alone did not ($p = 1.000$). These findings confirm that curing outcomes may depend on the extract concentration rather than the specific antibiotic used.

The 33.33% curing rate observed aligns with reports from Yogini et al. (2015), who recorded similar outcomes using *Cuminum sativum*. Streptomycin resistance was 100% reversed post-curing, while rifampin resistance persisted, except in 4 isolates (33.33%) that shifted from resistance to intermediate or sensitive status (zones of 16–18 mm). This suggests that increased or optimized concentrations may improve curing efficacy.

The overall plasmid curing potential of *N. sativa* (n-hexane extract) ranged from 33.33% to 90.9% across the tested concentrations. These findings are consistent with prior studies (Yogini et al., 2015; Maryam et al., 2016), which reported natural extract-based curing potentials between 33% and 100%. The variation in response among antibiotics suggests that curing efficiency may be both antibiotic-specific and concentration-dependent.

Conclusion

The curing study revealed a positive outcome from the use of *Nigella sativa* extracts in reversing the resistance of *S. aureus*, demonstrating the promising potential of *N. sativa* seed extract against multidrug-resistant *S. aureus*. Following treatment with *N. sativa*, the reassessed antibiogram indicated resistance reversal to levofloxacin (100%) and amoxicillin in 5 isolates (47.67%), showing a high response. The post-curing treatment of *S. aureus* with *N. sativa* resulted in increased sensitivity to levofloxacin in 11 isolates (91.67%) and to amoxicillin in 4 isolates (33.33%).

The findings of this study provide insight into the role of plasmids and resistance genes in multidrug-resistant pathogens, and highlight the potential of plant-based agents in combating plasmid-mediated resistance. Statistical analysis confirmed that the concentration of the extract plays a critical role in enhancing bacterial susceptibility following curing. These findings underscore the therapeutic value of *N. sativa* as a natural, safe, and effective alternative to synthetic curing agents such as acridine orange, which pose mutagenic risks. This approach could offer a novel and sustainable solution for restoring the efficacy of conventional antibiotics.

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